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Detection of Norwalk Virus in Stools by Enzyme Immunoassay

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The development of a solid-phase microtiter enzyme immunoassay (EIA) for detection of Norwalk virus antigen in stool samples is described. The EIA was compared with a previously developed radioimmunoassay (RIA) for detection of Norwalk virus antigen in stools obtained from 30 volunteers who received Norwalk virus. The EIA detected viral antigen in stools from 17 of the volunteers and the RIA detected viral antigen in 15. Seroconversion was a more sensitive indicator of infection in some patients. However, two samples from volunteers who were clinically ill but did not show seroconversion to Norwalk virus were positive for Norwalk virus antigen by both immunoassays. This indicates that antigen detection may be important for use in epidemiological studies. Neither of the immunoassays gave positive reactions for stools known to contain enteric adenovirus, rotavirus, or Hawaii virus, or in stools from patients with acute diarrhea of unknown cause. The stability of the EIA reagents and ease of use should provide a means for more extensive testing for Norwalk virus in outbreaks of gastroenteritis.

Key words: Norwalk virus, viral gastroenteritis, radioimmunoassay, enzyme immunoassay

INTRODUCTION

Norwalk virus was the first viral gastroenteritis agent of medical importance to be described [Dolin et al, 1972]. Disease caused by this agent was shown to be transmitted serially through human volunteers by the oral administration of bacteria-free and toxin-free stool filtrates derived initially from an adult involved in an outbreak of gastroenteritis in Norwalk, Ohio [Blacklow et al, 1972]. Although the virus has been partially characterized by its extraction from stools [Kapikian et al, 1973], lack of a suitable animal host or an in vitro cultivation method has limited the availability of reagents for study of this agent. By use of immune electron microscopy (IEM), the virus is known to be 27 nm in diameter [Kapikian et al, 1972]. It can also be detected in stools by radioimmunoassay (RIA) [Greenberg et al, 1978; Blacklow

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et al, 1979]. Both the RIA and IEM techniques require the use of human clinical materials as the source of both viral antigen and antibody, because it has not been possible to purify Norwalk virus antigen from stools sufficiently to permit preparation of immune sera or monoclonal antibody.

The use of RIA for detection of Norwalk virus antibody or antigen has provided a valuable technique for defining a major role of Norwalk virus in outbreaks of gastroenteritis [Greenberg et al, 1979; Kaplan et al, 1982; Cukor and Blacklow, 1984], but is subject to the limitations of radiolabeled reagents. In our laboratories, a particular problem has been the rapid loss of reactivity of ^{125}I-labeled anti-Norwalk virus IgG, such that assays need to be completed within two to three days after preparation of the radiolabeled IgG to obtain optimal sensitivity. To avoid this difficulty and to provide reagents that could be used more easily by others, we adapted the immunoassay to an enzyme-linked format.

In this report, we describe the development of an enzyme immunoassay (EIA) for detection of Norwalk virus antigen, and compare it with a previously reported RIA [Blacklow et al, 1979] for detection of Norwalk virus in the stools of adult volunteers who were exposed to the virus.

MATERIALS AND METHODS

Serum Specimens

Paired serum samples obtained from volunteers before and from two to six weeks after oral administration of Norwalk virus have been previously described [Blacklow et al, 1979]. Paired serum samples were also provided by G.W. Gary, Centers for Disease Control, Atlanta, Georgia.

Stool Specimens

A diarrheal stool specimen collected from a volunteer experiencing Norwalk virus illness and which was known to contain Norwalk virus by IEM was used as the positive control for RIA and EIA tests. This sample and most of the other test stool samples were from volunteers previously studied [Blacklow et al, 1979; Parrino et al, 1977; Schreiber et al, 1973; Widerlite et al, 1975]. Additional stool samples from eight volunteers inoculated with Norwalk virus were provided by G.W. Gary, Centers for Disease Control, Atlanta, Georgia. Six stools from volunteers experimentally inoculated with Hawaii virus were from those described [Schreiber et al, 1974]. Five stools from patients with diarrhea that contained adenovirus as shown by electron microscopy (EM) were provided by P.L. Ogra, State University of New York, Buffalo, and by P. Echeverria, Bangkok, Thailand. These samples contained virus that could be cultivated in Graham 293 cells but not in HEp-2 cells and were considered to contain enteric adenovirus serotypes. Ten stools containing rotavirus were samples previously found by us to contain rotavirus by EM and EIA [Herrmann et al, 1985]. Twenty-five stool samples from patients with naturally occurring acute diarrhea of unknown etiology were obtained from samples sent to our clinical laboratories and had been found to be negative for rotavirus as well as routine bacterial and parasitic pathogens. All stool specimens were stored at −70°C until use.

Immunoassays

Antigen detection. Both RIA and EIA tests were done in polyvinyl chloride microtiter plates (Cooke Engineering Co., Alexandria, VA). Wells of the plates were

coated with 100 μl of pre- and postchallenge sera (three-week convalescent) from a volunteer infected with Norwalk virus. Sera were diluted 1:10,000 in 0.01 M phosphate-buffered saline (PBS). The wells were coated for 24 hours at room temperature, washed with PBS, and postcoated with 1% w/v bovine serum albumin in PBS for 24 hours at 4°C. Stool samples to be tested were prepared as 10% w/v suspensions in veal infusion broth containing 0.5% bovine serum albumin. Fifty microliters of stool suspension was added to the wells, in duplicate, containing pre- or postchallenge serum, and incubated for 18–24 hours at 4°C. The plates were washed five times with PBS and detector antibody was added. The antibody used for detection was the IgG fraction of convalescent serum from a Norwalk virus infected volunteer different from the volunteer serum used as capture antibody. For the RIA, each well received 50 μl of ^{125}I-labeled antibody containing approximately 2×10^5 counts/min. For the EIA, each well received 50 μl of anti-Norwalk virus IgG labeled with peroxidase (Horseradish peroxidase, Sigma type VI, Sigma Chemical Co., St. Louis, MO) by the method of Wilson and Nakane [1978], which contained approximately 1 μg/ml IgG. The detector antibodies were incubated for two hours (EIA) or four hours (RIA) at 37°C. The wells were washed five times with PBS (RIA) or with PBS containing 0.1% v/v Tween-20. All washings were done with an automatic plate washer (Skatron Inc., Sterling, VA). For the RIA, wells were removed with a hole punch and bound radioactivity was determined in a gamma spectrometer. For the EIA, 100 μl of enzyme substrate (O-phenylene-diamine plus H_2O_2 in citrate-phosphate buffer, Abbott Laboratories, North Chicago, IL) was incubated in the wells for ten minutes at room temperature, and the reaction stopped with an equal volume of 1 N H_2SO_4. The amount of substrate hydrolysis was determined in a plate reader at A_{490} (Dynatech Laboratories, Alexandria, VA). For the RIA, the average of the counts bound to the wells coated with postchallenge serum was divided by the average of the counts bound to the wells coated with prechallenge serum. A ratio ≥ 2 was considered positive for Norwalk virus antigen. For the EIA, an A_{490} value for the wells coated with postchallenge serum that was at least 0.1 and that was greater than or equal to twice the A_{490} value for the wells coated with prechallenge serum was considered positive for Norwalk virus antigen.

Antibody detection. To determine seroconversion in acute and convalescent serum samples from volunteers inoculated with Norwalk virus, a blocking RIA was done. The procedure and sources of acute and convalescent sera were those previously described by us [Blacklow et al, 1979].

RESULTS

Sensitivity of Immunoassays

To determine the relative sensitivity of the RIA and EIA for detecting Norwalk virus antigen in stool material, the stool specimen used as a positive control was serially diluted in veal infusion broth with 0.5% w/v bovine serum albumin and tested by both immunoassays. The results are shown in Figure 1. Both of the immunoassays showed equal sensitivity for antigen detection in this stool sample and were able to detect Norwalk virus at a 1:5,120 dilution of the stool sample.

Antigen Detection in Stool Specimens

The sensitivity and specificity of each immunoassay was tested with stool specimens from volunteers inoculated with Norwalk virus and with additional stool

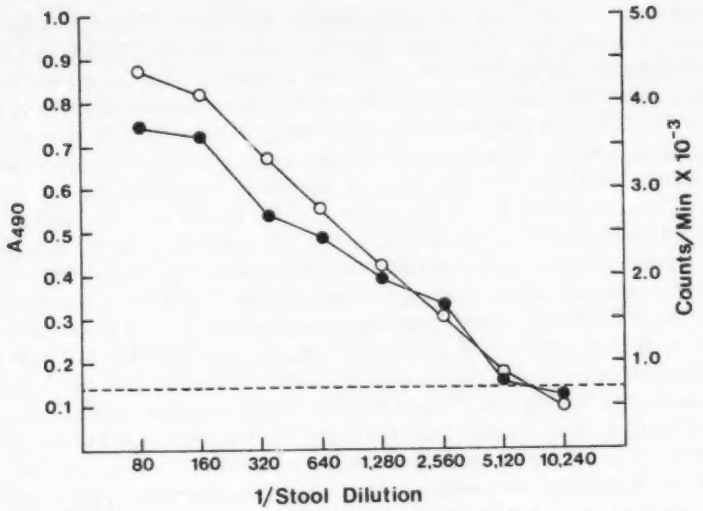

Fig. 1. Titration of Norwalk virus antigen in stool specimen from a volunteer by EIA (closed circles) and RIA (open circles). Points above the dotted line represent positive values for the immunoassays.

TABLE I. Detection of Norwalk Virus in Stools by Immunoassay

Patient group	No. of patients	No. immunoassay positive[a]	
		RIA	EIA
Volunteers inoculated with Norwalk virus	30	15	17
Volunteers inoculated with Hawaii virus	6	0	0
Enteric adenovirus-infected	5	0	0
Rotavirus-infected	10	0	0
Diarrhea of unknown etiology	25	0	0

[a]One patient positive by RIA was negative by EIA; 14 were positive by both immunoassays.

samples from naturally occurring cases of gastroenteritis owing to other viral agents or unknown causes. The results are shown in Table I. Of the 30 volunteers who received Norwalk virus, 15 had antigen in their stools detectable by RIA, and 17 had Norwalk antigen detectable by EIA. For many of the 30 volunteers, several stools had been sequentially collected, from the time of or before virus administration to postillness, if illness occurred. As a result, there were a total of 132 stool specimens available for testing. Of these, 41 were found positive for Norwalk virus antigen by EIA, 26 by RIA, with 18 positive by both assays. There were 83 samples negative by both tests. Both of the immunoassays showed high specificity for Norwalk virus and no preinoculation stools were positive by RIA or EIA. There were no positive results obtained with either immunoassay in stools collected from patients known to be infected with enteric adenovirus, rotavirus, Hawaii virus, or in stools from patients with acute diarrhea of unknown cause.

Correlation of Immunoassays With Patient Response

Clinical illness. Administration of Norwalk virus to volunteers may or may not result in the clinical symptoms of Norwalk virus gastroenteritis. The two immunoassays were compared for sensitivity in detecting antigen in stools from symptomatic or asymptomatic volunteers (Table II). Both immunoassays detected antigen more frequently in symptomatic patients than in asymptomatic volunteers, and the EIA appeared to be more sensitive than the RIA for detecting viral antigen in symptomatic patients.

Seroconversion. Seroconversion has been the standard technique used to determine Norwalk virus infection in epidemiological studies. As is shown in Table III, the immunoassays were more likely to detect antigen in stools from patients who demonstrated seroconversion, but they also detected viral antigen in stools from patients who did not show a serological response to the virus. Of the eight patients who did not show seroconversion, four were clinically ill. Two of these ill patients were positive for Norwalk virus antigen by both immunoassays, and a third patient was positive by RIA only. The EIA appeared to show an overall higher sensitivity in patients who seroconverted to Norwalk virus.

DISCUSSION

The EIA test to detect Norwalk virus antigen that we have developed is equivalent in sensitivity to the RIA test as indicated by titration of virus-containing

TABLE II. Correlation of Immunoassay Results With Clinical Illness in Norwalk Virus Inoculated Volunteers

Patient group	No. of patients	No. immunoassay positive[a]	
		RIA	EIA
Symptomatic	24	13	16
Asymptomatic	6	2	1

[a]All symptomatic patients positive by RIA were positive by EIA; the asymptomatic patient positive by EIA was positive by RIA.

TABLE III. Correlation of Immunoassay Results With Seroconversion in Norwalk Virus Inoculated Volunteers

Patient group	No. of patients	No. immunoassay positive[a]	
		RIA	EIA
Seroconversion positive	15	7	10
Seroconversion negative	8	3	2

[a]Seroconversion positive patients positive by RIA were also positive by EIA; seroconversion negative patients positive by EIA were also positive by RIA.

stool material. However, as measured by detection of Norwalk virus antigen in stools from symptomatic volunteers, the EIA test appeared to be more sensitive than the RIA procedure. The major advantage of the EIA test, however, is the stability of the reagents. As noted above, the ^{125}I-labeled anti-Norwalk virus IgG begins to lose reactivity two to three days after preparation. In contrast, portions of the same IgG fraction labeled with peroxidase showed no loss of reactivity after six months of storage at 4°C (data not shown). Thus, it is far easier to provide a consistent assay with the EIA, which also can be performed in more convenient settings than in laboratories with radioactive materials and equipment.

It was interesting to note that some of the stool samples positive by both immunoassays were from volunteer patients negative for Norwalk virus infections by seroconversion. Because the stool samples were from patients who received Norwalk virus and were clinically ill, there is little possibility that the immunoassay results were spurious. Thus, antigen detection is a more sensitive means for diagnosing infection than serology for some patients, much as the converse is also true.

Detection by RIA of Norwalk virus in stools from naturally occurring Norwalk virus outbreaks, however, has been of low efficiency to date [Grohmann et al, 1980; Gunn et al, 1980] such that serological methods have been needed for all of the epidemiological studies reported. The ability to provide a reliable antigen detection method that can be more easily provided to others would enable a greater number of stool samples to be routinely tested for Norwalk virus antigen. The EIA procedure we describe here should permit rapid testing of stool samples for Norwalk virus epidemiological studies.

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